



Quercetin attenuates neuronal cells damage in a middle cerebral artery occlusion animal model

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ABSTRACT. Cerebral ischemia is a neurological disorder with high mortality. Quercetin is a flavonoid compound that is abundant in vegetables and fruits. It exerts anti-inflammatory and anti-apoptotic effects. This study investigated the neuroprotective effects of quercetin in focal cerebral ischemia. Male Sprague-Dawley rats were subjected to middle cerebral artery occlusion (MCAO) to induce focal cerebral ischemia. Quercetin or vehicle was injected 30 min before the onset of ischemia. A neurological function test, brain edema measurement, and 2,3,5-triphenyltetrazolium chloride staining were performed to elucidate the neuroprotective effects of quercetin. Western blot analysis was performed to observe caspase-3 and poly ADP-ribose polymerase (PARP) protein expression. MCAO leads to severe neuronal deficits and increases brain edema and infarct volume. However, quercetin administration attenuated the MCAO-induced neuronal deficits and neuronal degeneration. We observed increases in caspase-3 and PARP protein levels in MCAO-operated animals injected with vehicle, whereas quercetin administration attenuated these increases in MCAO injury. This study reveals the neuroprotective effect of quercetin in an MCAO-induced animal model and demonstrates the regulation of caspase-3 and PARP expression by quercetin treatment. These results suggest that quercetin exerts a neuroprotective effect through preventing the MCAO-induced activation of apoptotic pathways affecting caspase-3 and PARP expression.

KEY WORDS: caspase-3, neuroprotection, PARP, quercetin

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Ischemic stroke including cerebral ischemia and cardiac ischemia is one of the leading causes of mortality and disability [9, 10]. Cerebral ischemic injury is associated with multiple mechanisms involved in the ischemic process, and ultimately leads to neuronal death and neurologic impairment via various signaling pathways [29]. In particular, oxidative stress is a major pathway that leads to neuronal cell death [34]. Quercetin is a natural flavonoid mainly contained in fruits, vegetables, and teas. It has a flavone nucleus molecular structure that includes two benzene rings linked by a heterocyclic pyrone ring [6]. This compound performs various functions including free radical scavenging and has anti-inflammatory, anti-coagulant, and anti-ischemic properties [1, 35]. Quercetin has been found to attenuate histopathological changes in a brain ischemia model and exert neuroprotective effects [4, 31]. Moreover, quercetin prevents the disruption of the blood-brain barrier during cerebral focal ischemia through reducing the elevated level of matrix metalloproteinase-9, consequently improving functional outcomes [20]. Quercetin, a powerful antioxidant, also prevents free radical generation and protects brain tissue against oxidative stress-associated damage in focal cerebral ischemia [1]. However, the neuroprotective mechanisms of quercetin are very complex and have not been fully elucidated. This study investigates the neuroprotective effect of quercetin and its apoptotic signal regulation in focal cerebral ischemia.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (200–220 g, $n=32$) obtained from Samtako Co. (Animal Breeding Center, Osan, Korea) were divided into the following groups: vehicle + sham, quercetin + sham, vehicle + middle cerebral artery occlusion (MCAO), and quercetin + MCAO (8 rats per group). Quercetin (30 mg/kg, Sigma, St. Louis, MO, U.S.A.) was dissolved in 0.05% dimethyl sulfoxide (DMSO) in phosphate buffered saline and administrated to animals by intraperitoneal injection 1 hr before the onset of ischemia [12, 28]. Vehicle-treated animals were administrated with DMSO solution only. Experimental animals were housed in a

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temperature controlled room (25°C) with artificially adjusted light conditions (12-hr light/12-hr dark cycle). Animals were supplied with water and food *ad libitum*. All experimental procedures were performed in accordance with the approved guidelines of the Institutional Animal Care and Use Committee of Gyeongsang National University.

Middle cerebral artery occlusion

MCAO surgical procedure was performed to induce focal cerebral ischemia based on a previously described method [15, 23]. Animals were anesthetized by an intra-muscular injection of Zoletil (50 mg/kg; Virbac, Carros, France). After a midline incision was made in the neck, the right common carotid artery was isolated from the adjacent muscles and nerves. The right common carotid artery was then ligated with a vascular clip and the external carotid artery was cut. A 4/0 monofilament nylon suture with flame-rounded tip by heating was inserted from the stump of the external carotid artery into the internal carotid artery until slight resistance was felt. A blunt tip of the intraluminal filament was placed in the middle cerebral artery approximately 2.5 cm from the bifurcation of the common carotid artery. In sham-operated animals, the surgical procedures were performed without insertion of the nylon filament. The neck was closed with nylon sutures and animals were kept on a heating pad to maintain body temperature. Animals were returned to their cages, given free access to food and water, and sacrificed 24 hr after the MCAO surgical operation. A previous study demonstrated that infarct volume and neurological function score were significantly impaired 24 hr after MCAO operation [16]. Moreover, anti-apoptotic effect of quercetin was significantly increased 24 hr after MCAO induction [21]. Thus, we collected brain tissue 24 hr after MCAO.

Neurological function tests and edema measurements

Neurological function tests were performed 24 hr after the MCAO procedure. Neurological function was evaluated according to a five-point scoring system: no deficit (0), normal posture but failure to extend the forepaw on the contralateral side of the ischemic region (1), normal posture but circling to the contralateral side of the ischemic region (2), falling to the contralateral side of the ischemic region (3), or no spontaneous movement (4) [17, 23, 32]. To measure edema in the cerebral cortex, the right cerebral cortex was immediately isolated from brain tissue and its weight was measured as the wet weight. After the wet weight measurement, the right cerebral cortex was dried at 100°C for 24 hr, and the weight of the dried tissue was measured as the dry weight. To determine the index of cerebral cortex edema, the brain water content (%) was calculated as follows: [(wet weight–dry weight)/wet weight] × 100 [17, 32].

Triphenyltetrazolium chloride staining

Brain tissues were quickly removed and sliced into 2 mm coronary sections using a brain matrix (Ted Pella, Redding, CA, U.S.A.). The section level was marked with bregma level [26]. Sliced brain tissues were immersed at 37°C for 20 min in 2% triphenyltetrazolium chloride (TTC; Sigma) and fixed in 10% formalin for 24 hr. Fixed tissues were scanned by an Agfa ARCUS 1200™ (Agfa-Gevaert, Mortsel, Belgium) and stained images were analyzed using Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, U.S.A.) in order to evaluate the infarct volume. The proportion of the ischemic area (%) was determined by the following formula: infarction area/whole section area × 100.

Hematoxylin and Eosin staining

Brain tissues were fixed in 4% formaldehyde with 0.1 M phosphate buffered saline) and washed with tap water. Tissues were dehydrated by series of graded ethyl alcohol from 70 to 100%), cleaned with xylene, embedded in paraffin using embedding center (Leica, Westlar, Germany). Paraffin blocks were cut into 4 μm sections and sections were placed on glass slides. Sections were deparaffinized with xylene and hydrated by graded with ethyl alcohol series from 100 to 70%. Sections were stained with Harris' hematoxylin solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 3 min and Eosin Y (Sigma-Aldrich) for 1 min. Sections were washed with tap water, dehydrated with graded ethyl alcohol series, mounted with permount mounting solution (Thermo Fisher Scientific, Waltham, MA, U.S.A.), photographed using Olympus microscope (Olympus, Tokyo, Japan).

Fluoro-Jade B staining

Fluoro-Jade B staining was performed to examine the degeneration of neurons after focal brain ischemia. Brain sections were de-paraffinized with xylene and rehydrated with ethyl alcohol. Subsequently, sections were incubated with 1% sodium hydroxide in 80% ethyl alcohol, 70% ethyl alcohol, and distilled water. Sections were reacted with a 0.06% potassium permanganate solution for 10 min and stained with a 0.1% acetic acid solution containing 0.01% Fluoro-Jade B for 30 min. After staining, sections were rinsed with distilled water and dried on a slide warmer. Dried sections were incubated in 4',6-diamidino-2-phenylindole (DAPI, Sigma) and mounted with dibutylphthalate polystyrene xylene (DPX) mounting media (Sigma). Section images were observed and captured with a confocal laser scanning microscope (FV 1000, Olympus). The proportion of Fluoro-Jade B positive cells was measured by the ratio of Fluoro-Jade B positive cells to DAPI positive cells.

Western blot analysis

Right cerebral cortex tissues were isolated from brain for Western blot analysis. For detection of PARP and caspase-3 proteins expression, Western blot analysis was performed as a previously described method [18]. Proteins were extracted with lysis buffer [1% Triton X-100, 1 mM EDTA in 1 × PBS (pH 7.4)] containing 200 μM phenylmethylsulfonyl fluoride, and the concentrations of isolated proteins were measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.). Protein samples (30 μg)

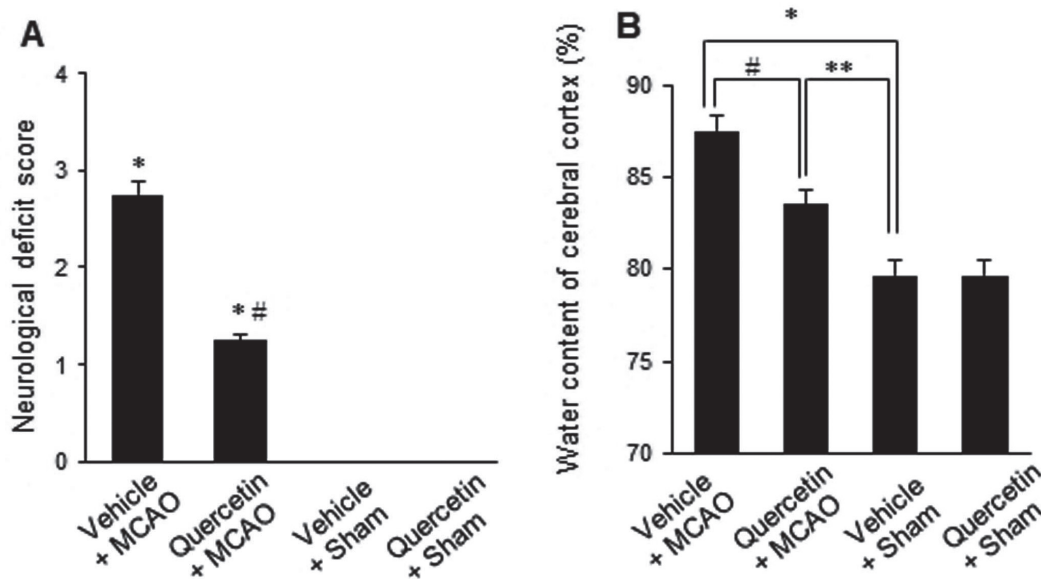


Fig. 1. Neurobehavioral scores (A) and edema measurement (B) in vehicle + sham, quercetin + sham, vehicle + middle cerebral artery occlusion (MCAO), and quercetin + MCAO animals. Quercetin attenuated the neurological deficits and edema value induced by ischemic stroke. Data ($n=4$) are represented as the mean \pm S.E.M. * $P<0.01$, ** $P<0.05$ vs. vehicle + sham animals, # $P<0.05$ vs. vehicle + MCAO animals.

were denatured with loading buffer by heating at 100°C for 3 min and cooled on ice. Protein samples were separated through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidenedifluoride membrane (Millipore, Billerica, MA, U.S.A.). The membrane was incubated in 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hr to block nonspecific antibody binding. After washing three times with TBST for 10 min, the membrane was incubated with the following primary antibodies: anti-PARP, anti-caspase-3 (1:1,000, Cell Signaling Technology, Beverly, MA, U.S.A.), and anti- β -actin (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) overnight at 4°C. Membranes were washed with TBST and incubated with the following secondary antibodies: horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:5,000, Cell Signaling Technology) for 2 hr. After washing with TBST, the membranes were incubated with enhanced chemiluminescence detection reagents (GE Healthcare, Little Chalfont, U.K.) according to the manufacturer's protocol, and visualized on Fuji medical X-ray film (Fuji Film, Tokyo, Japan).

Data analysis

All experimental data are represented as the mean \pm S.E.M. Intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, U.S.A.). The results for each group were compared by two-way analysis of variance (ANOVA) followed by Scheffé's *post hoc* test. Differences were considered significant at $P<0.05$.

RESULTS

As shown in Fig. 1, focal cerebral ischemic injury leads to severe neurological behavioral deficits and cerebral edema. Neurological deficits ranging from occasional rotating movements to spontaneous circling appeared in vehicle + MCAO-induced animals. Quercetin treatment attenuated the MCAO-induced neurological deficits and showed only a mild focal neurologic deficit of failure to fully extend the left forepaw. However, obvious signs of neurological deficits were not observed in sham-operated animals. Neurological function scores were 2.75 ± 0.25 and 1.32 ± 0.21 in vehicle + MCAO and quercetin + MCAO animals, respectively (Fig. 1A). MCAO ischemic injury led to severe cerebral edema, whereas quercetin treatment attenuated this change. The water content of the cerebral cortex was 87.53 ± 2.75 and $83.14 \pm 2.12\%$ in vehicle + MCAO and quercetin + MCAO animals, respectively (Fig. 1B).

TTC staining demonstrated a significant increase in the infarct volume in MCAO-induced animals (Fig. 2A). However, quercetin treatment alleviated this increase caused by MCAO injury. Infarct volumes were 28.95 ± 3.24 and $18.54 \pm 2.1\%$ in vehicle + MCAO and quercetin + MCAO animals, respectively (Fig. 2B). Sham-operated animals did not have any infarct regions in both vehicle-treated and quercetin-treated groups. Figure 3 showed the morphological changes of cerebral cortex using Hematoxylin and Eosin staining. MCAO animal with vehicle had numerous vacuoles in the cytoplasm of the pyramidal cells, and condensed and shrunken nuclei. Moreover, most of the dendrites were shrunk and lost. However, quercetin treatment alleviated MCAO-induced these damages. Vacuoles and condensed nuclei were decreased in quercetin + MCAO animals, and pyknotic nuclei were

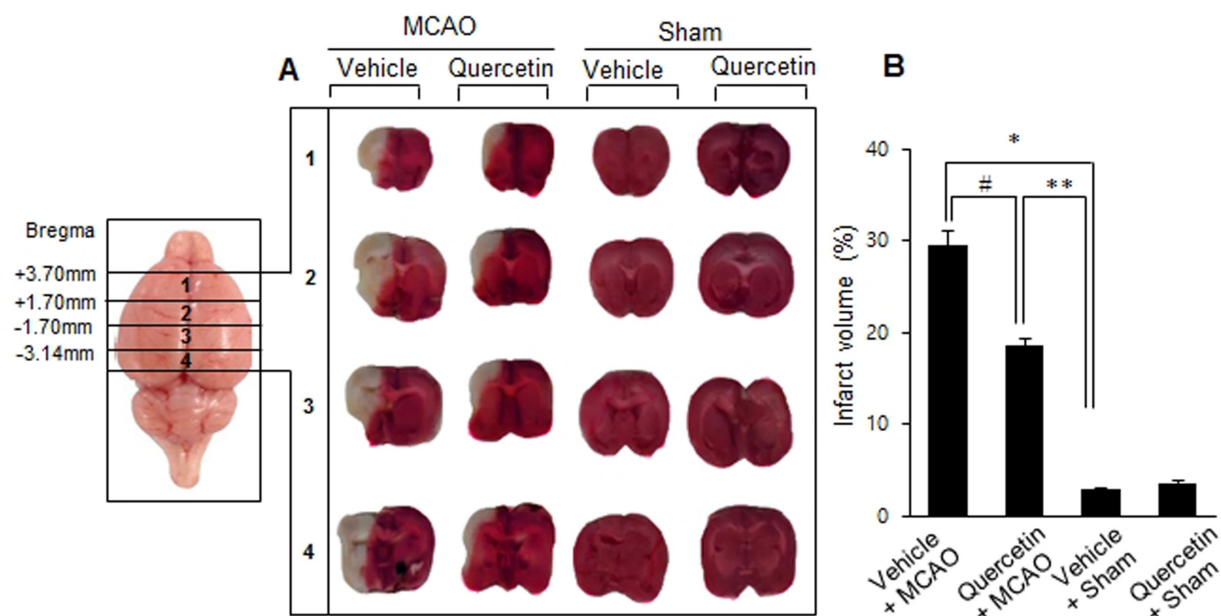


Fig. 2. Representative photograph of TTC staining (A) and infarct volume (B) in vehicle + sham, quercetin + sham, vehicle + middle cerebral artery occlusion (MCAO), and quercetin + MCAO animals. The ischemic area remained white, while the intact area was stained red (A). Infarct volume was calculated by the ratio of the infarction area to the total section area (B). Quercetin attenuated the MCAO-induced infarct region. Data ($n=4$) are represented as the mean \pm S.E.M. * $P<0.01$, ** $P<0.05$ vs. vehicle + sham animals, # $P<0.05$ vs. vehicle + MCAO animals.

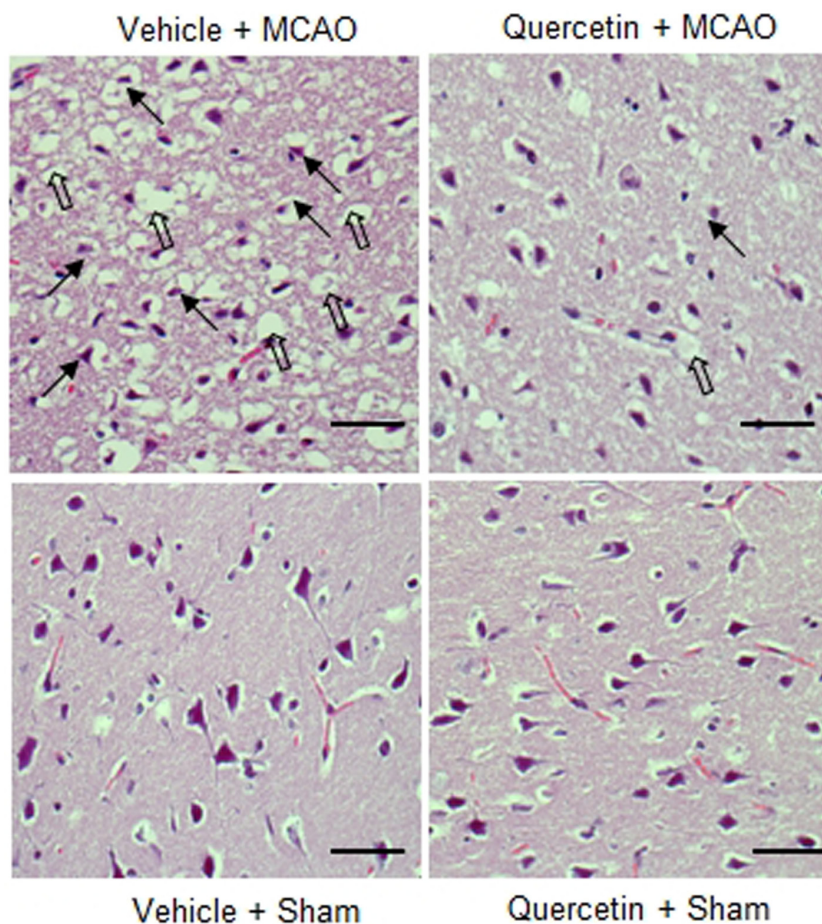


Fig. 3. Representative photograph of Hematoxylin and Eosin staining in vehicle + sham, quercetin + sham, vehicle + middle cerebral artery occlusion (MCAO), and quercetin + MCAO animals. Arrows indicate shrunken and condensed nuclei and open arrows indicate swelled and vacuolated forms. Scale bar=100 μ m.

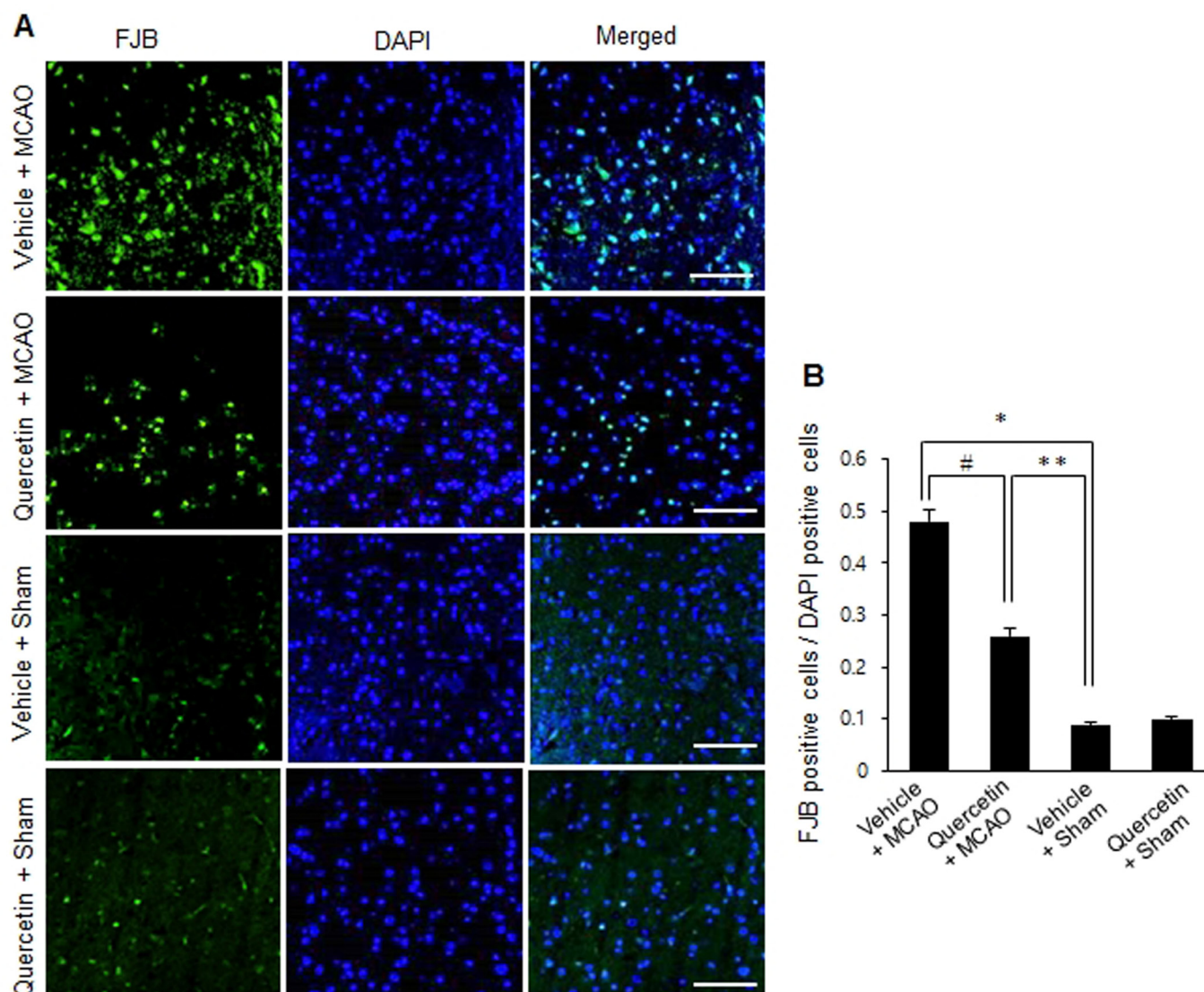


Fig. 4. Representative photos of Fluoro-Jade B and DAPI staining in the cerebral cortex of vehicle + sham, quercetin + sham, vehicle + middle cerebral artery occlusion (MCAO), and quercetin + MCAO animals. Many cells positive for Fluoro-Jade B staining were observed in animals with MCAO injury, while quercetin decreased the number of Fluoro-Jade B positive cells (A and B). Scale bar: 100 μ m. Data ($n=4$) are shown as the mean \pm S.E.M. * $P<0.01$, ** $P<0.05$ vs. vehicle + sham animals, # $P<0.05$ vs. vehicle + MCAO animals.

reduced. Length of dendrites was also recovered as that of sham operated animals. We observed round and large nucleus, and intact cytoplasm and dendrites in both vehicle + sham and quercetin + sham animals.

Fluoro-Jade B staining is an established marker for neuronal degeneration and indicates damaged neurons susceptible to cell death [30]. Fluoro-Jade B positive cells were observed in the ischemic region of vehicle + MCAO animals (Fig. 4A). However, quercetin treatment substantially diminished the number of Fluoro-Jade B positive cells in MCAO-operated animals. Sham operated animals did not have any Fluoro-Jade B positive cells regardless of vehicle or quercetin treatment. The levels of Fluoro-Jade B positive cells were 0.48 ± 0.07 and 0.26 ± 0.05 in the cerebral cortices of vehicle + MCAO and quercetin + MCAO animals, respectively (Fig. 4B).

Western blot analyses showed changes in PARP and caspase-3 expression levels in MCAO-operated animals in response to quercetin treatment (Fig. 5A). PARP and caspase-3 protein levels were increased in vehicle + MCAO animals compared to sham-operated animals. However, the increase of these proteins in MCAO-operated animals was attenuated in the cerebral cortex of quercetin-treated animals. PARP levels were 1.28 ± 0.07 and 0.98 ± 0.05 in the cerebral cortices of vehicle + MCAO and quercetin + MCAO animals, respectively (Fig. 5B). Moreover, Caspase-3 levels were 1.12 ± 0.08 and 0.42 ± 0.03 in the cerebral cortices of vehicle + MCAO and quercetin + MCAO animals, respectively (Fig. 5C).

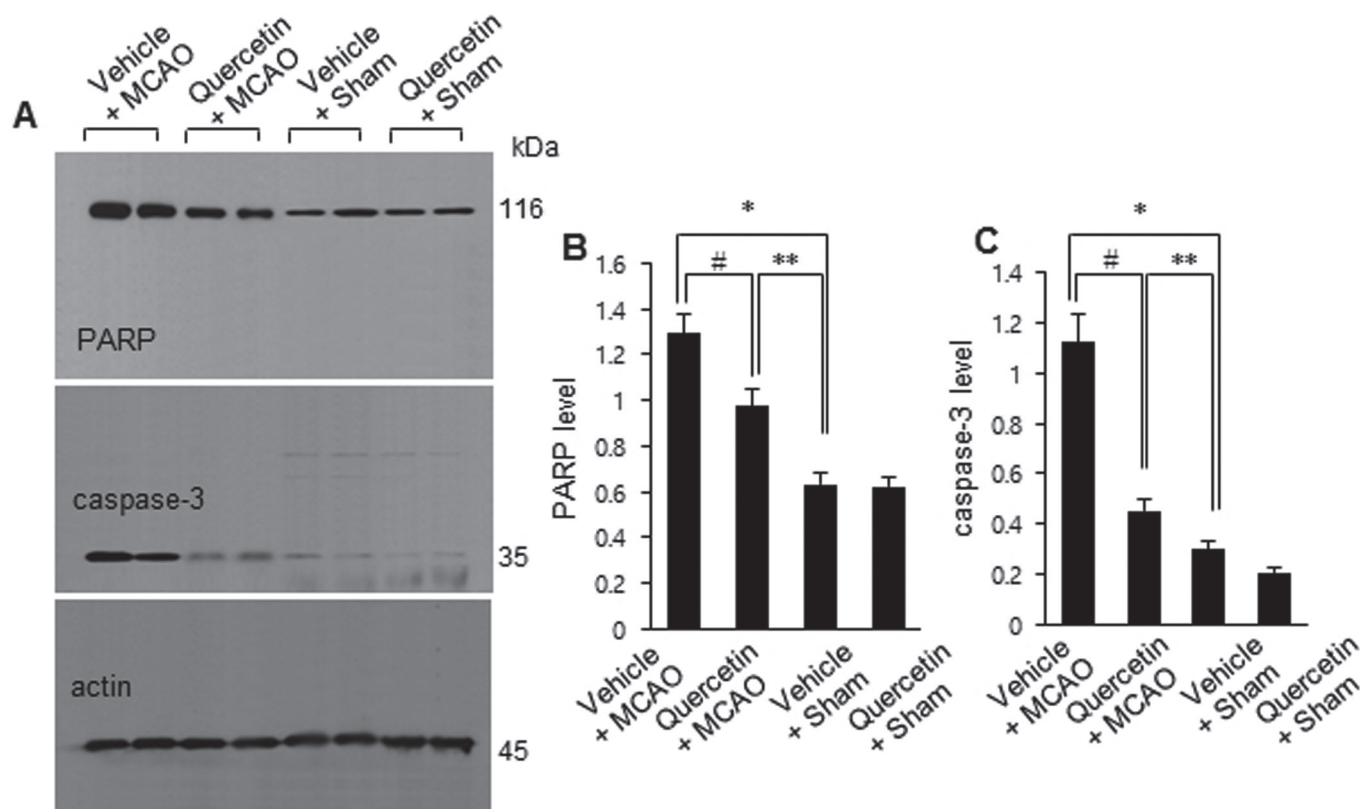


Fig. 5. Western blot analysis (A–C) of PARP and caspase-3 in the cerebral cortex of vehicle + sham, quercetin + sham, vehicle + middle cerebral artery occlusion (MCAO), and quercetin + MCAO animals. Densitometric analysis is represented as a ratio of PARP (B) and caspase-3 (C) staining intensity to actin intensity. Data ($n=4$) are shown as the mean \pm S.E.M. * $P<0.01$, ** $P<0.05$ vs. vehicle + sham animals, # $P<0.05$ vs. vehicle + MCAO animals.

DISCUSSION

It is known that cerebral ischemia in rats leads to neurological movement disorders such as rotating movements to the contralateral side of the ischemic region or no spontaneous movement [17, 23, 32]. Moreover, pathological change with brain edema causes following the cerebral ischemia and reperfusion in rats [32]. MCAO also leads to significant infarct volume and serious neuronal cells damage. Quercetin attenuated the severe neurological deficits and brain edema and significantly reduced the infarct volume caused by MCAO. Infarct volume in brain tissue is accepted a critical evidence in assessing the consequences of ischemic injury which leads to neurological impairment and neuronal damage. We used TTC staining methods to elucidate the morphological features of infarction following cerebral ischemic injury. Results of TTC staining clearly demonstrated that quercetin treatment attenuated infarct volume increase caused by MCAO injury. Quercetin reduces neurological behavioral deficits and infarct volume in focal cerebral ischemia. Moreover, quercetin prevented MCAO-induced degeneration of neuronal cells. MCAO increased the number of cells with positive staining for Fluoro-Jade B, a marker for degenerating neurons, while quercetin treatment decreased the number of Fluoro-Jade B positive cells. Thus, we demonstrated that quercetin exerts neuroprotective effects against MCAO-induced ischemic injury in an animal model. Quercetin exerts a variety of pharmacological activities for further clinical application. The safety and beneficial effects of quercetin has been elucidated in clinical trials. Quercetin is not carcinogenic and mutagenic toxicity [24]. Thus, its safety is approved in human. Moreover, quercetin exerts antioxidant and anticancer effects in clinical studies and controls blood pressure in hypertensive patients [2, 8]. Previous studies can suggest that that quercetin has a sufficient potential as a clinical therapeutic agent.

We confirmed preconditioning effect of quercetin in cerebral ischemia. Previous studies demonstrated that pre-treatment with quercetin results in marked reduction in infarct size and neurological deficits [1, 21]. Moreover, other study has shown pre- and post-conditioning effects on quercetin in MCAO. The results showed that quercetin treatment 30 min before and 4 hr after ischemia provided significant protection from ischemic injury by reducing the infarct volume and other neurological deficit. However, 30 min pre-condition of quercetin is more effective in improvement of neurological functional score and infarct volume in cerebral cortex than 2 and 4 hr post-condition of quercetin [25]. Thus, they suggest that quercetin pre-conditioning was more effective in improving neurological function and ischemic brain injury than post-conditioning [25]. In this study, we also showed that 30 min preconditioning of quercetin significantly improved the neurological function score and infarct volume.

The molecular mechanism of ischemic brain damage is characterized by a complicated pathophysiology. Brain ischemia leads to a cascade of events such as glutamate excitotoxicity, energy failure, and formation of toxic radicals [5, 14]. These events subsequently lead to neuronal cells death. The beneficial role of quercetin in ischemic brain injury is attributed to its antioxidant and anti-inflammatory profile [4, 13, 33]. Antioxidant and anti-inflammatory effects of quercetin determine nitric oxide production by electron paramagnetic resonance and inhibit NF-kappaB activations [13, 33]. Moreover, quercetin exerts a vasodilatory effect by Akt-independent and PKA-dependent mechanism [22]. Quercetin decreases blood pressure and reduces the severity of hypertensive in spontaneously hypertensive rats by improvement of endothelial function [3, 7, 19, 27]. Quercetin not only inhibits acid sensing ion channels mediated acidotoxicity but also prevents apoptosis through anti-apoptotic mechanisms based on the PI3K/Akt pathway in focal cerebral ischemia. PARP and caspase-3 are apoptosis-associated proteins which activate DNA fragmentation factors and cause DNA cleavage, and thus are used as indicators of apoptosis. PARP and caspase-3 activation induces apoptotic processes via a mitochondria-mediated apoptotic pathway. Activity of PARP and caspase-3 were elevated in cerebral cortex of MCAO group without quercetin. This study revealed that quercetin exposure alleviated increases in the expression of PARP and caspase-3 proteins in MCAO injury. Inhibition of PARP and caspase-3 by quercetin treatment protected neuronal cells against focal cerebral ischemia caused by MCAO injury. We verified that quercetin prevented PARP and caspase-3 activation in cerebral ischemic injury, and attenuated apoptotic cell death. Reduction of caspase-3 and PARP expression indicates an inhibition of apoptotic cell death leading to the prevention of neuronal cell damage. Thus, quercetin is contributed as PARP and caspase-3 inhibitors. Quercetin acts as an important agent which is associated with caspase-mediated cell death and cell dysfunction. PARP inhibition by pharmacological agents is mediated to prevention of apoptotic cell death [11]. Quercetin prevents neuronal damage in MCAO rats, which is attributed to inhibition of neurological deficit, PARP activity, and caspase-3 activity. This study clearly demonstrated the neuroprotective effects of quercetin in a cerebral ischemic animal model. Moreover, we found that quercetin regulates PARP and caspase-3 activation. These findings suggest that quercetin protects neuronal cells against cerebral ischemic damage via inhibition of the apoptotic pathway.

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